Chemistry–A European Journal
Research Article
doi.org/10.1002/chem.202302400
Chemistry Europe
European Chemical Societies Publishing
www.chemeurj.org

Special Collection

Non-Hydrolysable Analogues of Cyclic and Branched Condensed Phosphates: Chemistry and Chemical Proteomics

Tobias Dürr-Mayer,[a] Andrea Schmidt,[b] Stefan Wiesler,[a] Tamara Huck,[a] Andreas Mayer,[b] and Henning J. Jessen*[a, c]

Dedicated to Evamarie Hey-Hawkins.

Studies into the biology of condensed phosphates almost exclusively cover linear polyphosphates. However, there is evidence for the presence of cyclic polyphosphates (metaphosphates) in organisms and for enzymatic digestion of branched phosphates (ultraphosphates) with alkaline phosphatase. Further research of non-linear condensed phosphates in biology would profit from interactome data of such molecules, however, their stability in biological media is limited. Here we present syntheses of modified, non-hydrolysable analogues of cyclic and branched condensed phosphates, called meta- and ultraphosphonates, and their application in a chemical proteomics approach using yeast cell extracts. We identify putative interactors with overlapping hits for structurally related capture compounds underlining the quality of our results. The datasets serve as starting point to study the biological relevance and functions of meta- and ultraphosphates. In addition, we examine the reactivity of meta- and ultraphosphonates with implications for their "hydrolysable" analogues: Efforts to increase the ring-sizes of meta- or cyclic ultraphosphonates revealed a strong preference to form trimetaphosphate-analogue structures by cyclization and/or ring-contraction. Using carbodiimides for condensation, the so far inaccessible dianhydro product of ultraphosphonate, corresponding to $P_4O_{11}^{2-}$, was selectively obtained and then ring-opened by different nucleophiles yielding modified cyclic ultraphosphonates.

Introduction

For a long time, cellular condensed phosphates were defined as exclusively linear structures[1,2] until evidence was recently given for the presence of cyclic polyphosphates (metaphosphates) in bacterial phosphate granules.[3] Studies before the 1970s – reporting metaphosphates in yeast – only caught little attention due to concerns about alterations of the structure and distribution of polyphosphates caused by the employed extraction and purification protocols.[3–7] While several biological functions[8] – depending on abundance, chain length and subcellular location – are known for linear polyphosphates and the enzymology of (poly)phosphate homeostasis has been well described for different pro- and eukaryotes,[9,10] the biology of metaphosphates remains understudied. However, evidence is available for their existence and a role in the origins of life has been discussed.[3,11] It was, for example, shown that hexametaphosphate is metabolized by *Xanthobacter autotrophicus* extracts but the catabolic enzyme(s) are unknown.[3] Studies on the interaction of metaphosphates with proteins are hampered by a dearth of water-stable, functionalized analogues, which allow the identification and enrichment of interactors.

In principle, several methods are known to activate trimetaphosphate by introduction of a leaving group (Scheme 1a), enabling functionalization by a subsequent nucleophilic reaction.[12–17] Considering larger ring-sizes, tetrametaphosphate esters are accessible from the reaction of a nucleophile with the anhydride $P_4O_{11}^{2-}$ as the activated form.[18–20] Avoiding the need for an additional activation step, aryne chemistry enables direct modification of metaphosphates with different ring-sizes representing a broadly applicable method.[21] Yet, metaphosphate esters are susceptible to nucleophiles, including water, and such reactions lead to linearization usually within minutes to hours, depending on the nucleophile. This reactivity is detrimental to their use as pull-down probes in proteomics studies.[12,13,15,22]

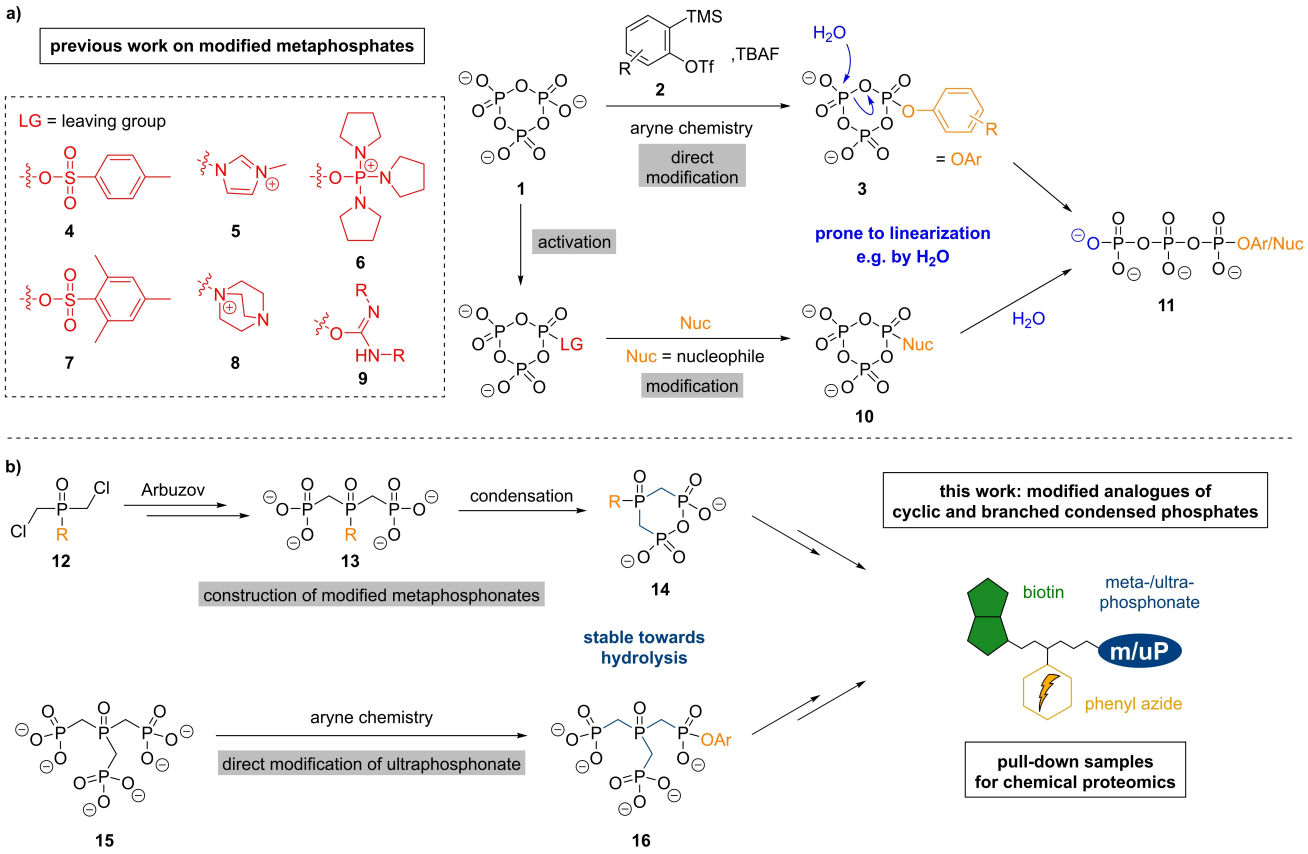
[a] Dr. T. Dürr-Mayer, Dr. S. Wiesler, T. Huck, Prof. Dr. H. J. Jessen
Institute of Organic Chemistry
Albert-Ludwigs-Universität Freiburg
Albertstraße 21, 79104 Freiburg im Breisgau (Germany)
E-mail: henning.jessen@oc.uni-freiburg.de

[b] A. Schmidt, Prof. Dr. A. Mayer
Département de Biochimie
Université de Lausanne
Chemin des Boveresses 155, CH-CH-1066 Epalinges (Switzerland)

[c] Prof. Dr. H. J. Jessen
Cluster of Excellence livMatS @ FIT – Freiburg Center for Interactive Materials and Bioinspired Technologies
Albert-Ludwigs-Universität Freiburg

Supporting information for this article is available on the WWW under https://doi.org/10.1002/chem.202302400

Special Collection Part of a Special Collection on the p-block elements.



Scheme 1. Approaches for modified metaphosphates and non-hydrolysable analogues of cyclic and branched condensed phosphates. a) Modification of metaphosphates using aryne chemistry or by activation with different leaving groups and subsequent nucleophilic reaction. Hydrolytic linearization of modified metaphosphates. b) Reaction design for modified meta- and ultraphosphonates employing Arbuzov and condensation reactions respectively aryne chemistry.

Phosphoric anhydride bonds can be stabilized via replacement of oxygen by CH_2 or CF_2 groups, which gives rise to phosphonates.[23,24,25] Taking advantage of the increased stability of such non-hydrolysable analogues, affinity reagents can be constructed to investigate the interactome of a substrate, as, for example, recently shown for inositol pyrophosphates.[26] A substitution of the ester function of modified trimetaphosphate by a stable P–C bond was recently reported by Cummins.[27] However, these trimetaphosphate analogues were usually linearized in water within 24 h, highlighting the need to stabilize phosphoanhydride bonds. Differently substituted bismethylene triphosphates are readily accessible using Arbuzov-type reactions.[28–30] Condensation of unmodified bismethylene triphosphate using *N*,*N*′-dicyclohexylcarbodiimide (DCC) yields the trimetaphosphate analogue, from here on called trimetaphosphonate, which Kenyon used to synthesize a non-hydrolysable adenosine triphosphate (ATP) analogue.[29] Since no simple coherent nomenclature is defined for differently CH_2-substituted analogues of condensed phosphates, we decided to generally indicate such modifications by naming structures as phosphonates, although they might contain a phosphinate (as in **14**) or phosphine oxide (as in **16**) substructure as well.

Based on the described results, we envisioned the synthesis of trimetaphosphonates modified at the phosphinate function rather than at the phosphonate subunits (Scheme 1b) and study the hydrolytic stability of this unexplored structural motif. Showing sufficient stability, trimetaphosphonate should be developed into capture compounds for pull-down experiments.

Orthophosphoryl metaphosphates (cyclic ultraphosphates) represent constitutional isomers of metaphosphates and were developed as potent polyphosphorylating reagents.[14,16,19,31–33] Due to the lability of the branching phosphate,[34] these structures can easily be linearized by different nucleophiles.[12,35] In the context of the hydrolysis of branched condensed phosphates (ultraphosphates), Van Wazer coined the anti-branching-rule in 1950, describing ultraphosphates as exceedingly unstable and more labile than cyclic or linear polyphosphates.[1,36] Over the decades, the rule persisted and led to the widespread perception that ultraphosphates will have no role to play in biology.[1,37] Even so, we know that many unstable modifications such as phosphohistidine[38] do exist in nature and therefore experimental approaches to discover a potential ultraphosphate biology are warranted. In this context, hydrolysis half-lives up to days were recently determined for synthetic monodisperse ultraphosphates and evidence was given for their hydrolysis by alkaline phosphatase.[39] The sensitivity of ultraphosphates towards acidic pH, nucleophilic reagents, divalent cations and drying however hamper their

enrichment and analysis in efforts to detect branched phosphates in biological samples. Thus, we aimed to functionalize a non-hydrolysable analogue, from here on called ultraphosphonate (**15**), to serve as a capture compound in a chemical proteomics approach to investigate the interactome of branched condensed phosphates for future studies into their potential biology.

Herein, we describe the syntheses of trifunctional pull-down probes with non-hydrolysable analogues of trimetaphosphate, different ultraphosphates as well as inorganic tetraphosphate as selection function. These capture compounds were applied in photoaffinity pull-down experiments with yeast cell extracts and putative interactors were identified. These hits may aid in the identification of enzymes that process or make metaphosphates or branched condensed phosphates.

Results and Discussion

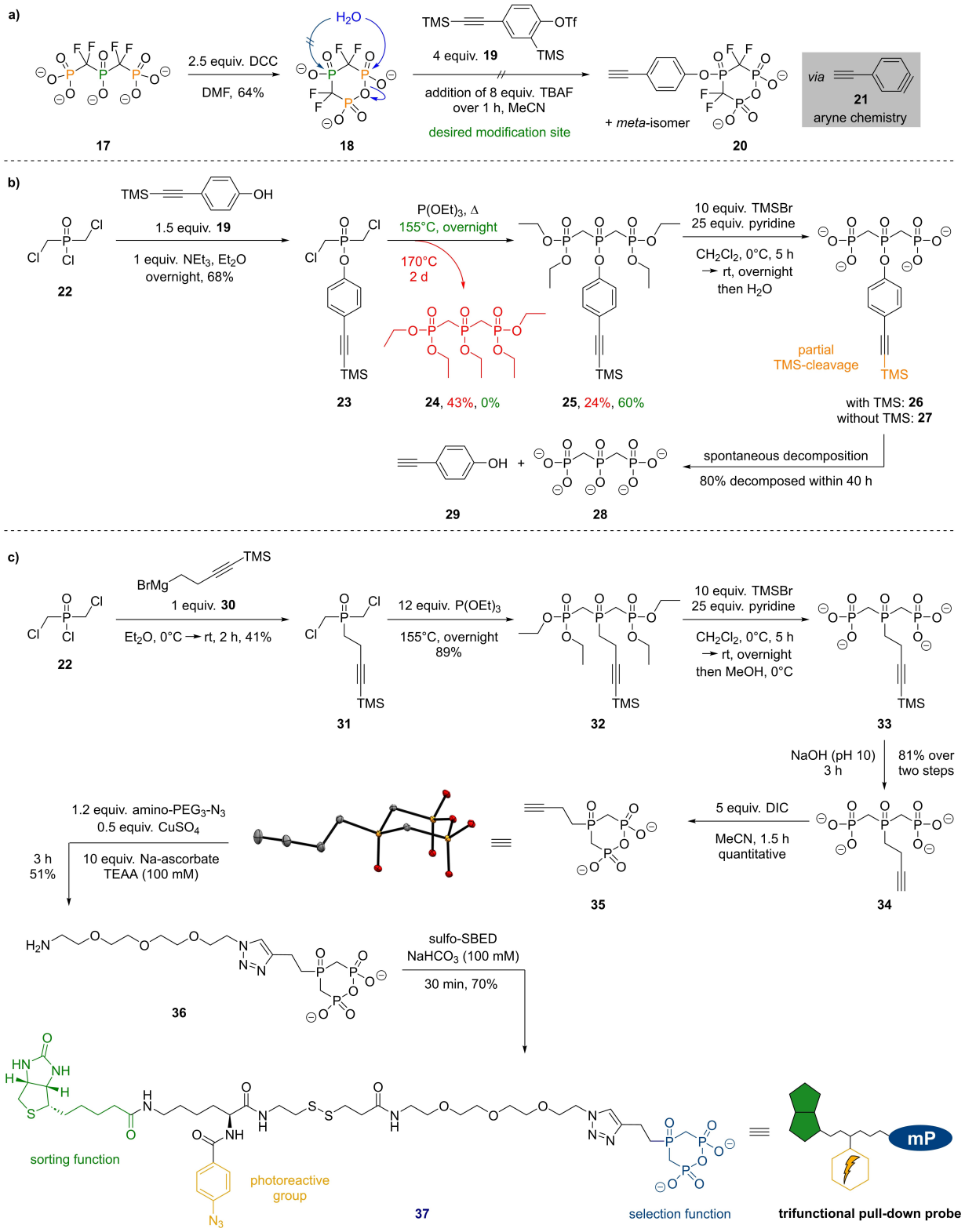
Synthesis of modified trimetaphosphonate

The aryne phosphate reaction provides direct access to arylated metaphosphates of controllable ring-size without the need for an additional activation step and was therefore initially tested as method to modify non-hydrolysable analogues of metaphosphates.[21] Since substitution of the bridging oxygen of a phosphoanhydride bond by a CF_2-group retains the correct polarity, while it is reversed with CH_2 as the bridging unit, bis(difluoromethylene)triphosphate (**17**) was synthesized following a procedure of Olah[24] and the corresponding tetrabutylammonium (TBA) salt of **17** cyclized in 64% yield using DCC for condensation (Scheme 2a). To avoid linearization of the esterified metaphosphate-analogue in accordance with previous reports,[12,13,15,21,27] arylation at the phosphinate oxygen was desired (Scheme 2a, marked in green). CF_2-trimetaphosphonate **18** was reacted with the alkynylated Kobayashi-type aryne precursor **19** to enable subsequent further functionalization by copper-catalysed 1,3-dipolar cycloaddition (CuAAC) resulting in a complex mixture instead of monoarylated **20**. The products were separated and characterized using HRMS and NMR analysis, revealing overreaction to bisarylated products, ring-opening by water or fluoride, products of a side reaction of the aryne with THF[21,40] and combinations thereof (see Supporting Information, Scheme S1). We therefore decided to first selectively introduce an alkyne moiety at the phosphinate function and then construct the bismethylene triphosphate using an Arbuzov reaction (Scheme 2b). The latter are usually performed with trialkylphosphites and the resulting phosphonate esters can be hydrolyzed by either refluxing with conc. HCl or treatment with TMSBr.[28,41] While strongly acidic conditions would likely hydrolyze phosphinate esters as well, reports on the selective removal of benzyl protecting groups in arylphosphate esters using TMSBr[42] and selective dealkylation of arylated bisphosphonates using TMSCl and NaI[43] suggested compatibility of these deprotection strategies.

Esterification of bis(chloromethyl)phosphinic chloride (**22**) with the alkyne-modified phenol **19** gave the arylphosphinate **23** in 68% yield. The Arbuzov reaction of **23** with $P(OEt)_3$ at 170 °C gave a mixture of aryl-modified **25** and pentaethyl bismethylene triphosphate (**24**) in 3:1 ratio overnight, as indicated by $^{31}P\{^1H\}$-NMR reaction control. This ratio changed to 2:3 after two days at 170 °C suggesting that **25** can arylate $P(OEt)_3$, similar to reports on alkylation of P(III) structures by phosphinate alkylesters.[44] This reactivity was not observed when the Arbuzov reaction was carried out at 155 °C, giving **25** in 60% yield. Of note, attempts to react bis(chloromethyl)phosphinic acid in an Arbuzov reaction without alkylation of the phosphinate function failed (see Supporting Information, Scheme S2). We found that the phosphinate is already alkylated after the first Arbuzov reaction and analyzed the corresponding product crystallographically (see Supporting Information, compound **S11**). Following the procedure of Guillaumet,[42] the ethyl esters of **25** were cleaved by treatment with a mixture of TMSBr and pyridine followed by hydrolysis of the silylated intermediate. Corresponding $^{31}P\{^1H\}$-NMR spectra of the reaction are shown in the Supporting Information, Scheme S3. ^{31}P-HMBC confirmed that the phosphinate still carried the aryl modification. Partial cleavage of the alkyne-TMS group was observed at pH 8.5, but albeit desilylation was desired for subsequent click-reactions, the pH was not further increased to avoid hydrolysis of the aryl ester under more basic conditions.[45] After strong anion exchange (SAX) purification, the aryl ester was even hydrolyzed at almost neutral pH with 80% of the isolated product being decomposed after 40 h in water. We therefore abandoned this synthetic strategy.

To avoid hydrolytic cleavage of the alkyne function, we reacted **22** with the Grignard reagent **30**[46] to access the phosphine oxide **31** in 41% yield (Scheme 2c).[47,48] The Arbuzov reaction of **31** with $P(OEt)_3$ gave **32** in 89% yield. The ethyl esters were cleaved by silylation using a mixture of TMSBr and pyridine[42] followed by methanolysis giving **33**. After treatment with NaOH solution (pH 10) to remove the alkyne-TMS group, **34** was obtained in 81% yield over two steps. After conversion to the TBA salt, **34** was condensed using *N*,*N*′-di*iso*propylcarbodiimide (DIC). $NaClO_4$ in acetone was used for precipitation giving product **35** in quantitative yield as sodium salt, which formed single crystals during slow evaporation. X-ray analysis confirmed the structure shown in Scheme 2c allowing comparison of bond lengths and angles of the non-hydrolysable analogue **35** with crystallographic data of trimetaphosphate tetramethylammonium salt.[49] While the P–O bond lengths in these two structures only differ by 1–2%, the P–C bond is markedly elongated to 1.81 Å compared with 1.65 Å for the P–O bonds in the trimetaphosphate salt. For the bond angles, deviations in the range of 5–13% were determined, corresponding to results of Yount for the comparison of pyrophosphate and its methylene-bridged analogue.[50]

We monitored the hydrolytic stability of the phosphoanhydride bond of **35** in water (neutral pH) at room temperature by $^{31}P\{^1H\}$-NMR. We did not detect any decomposition over the course of 24 h and found approximately 1% hydrolysis after ca. 6 days allowing further development of **35** into a capture compound for pull-down experiments. **35** was reacted with amino-PEG_3-azide in a CuAAC click reaction giving **36** in 51%

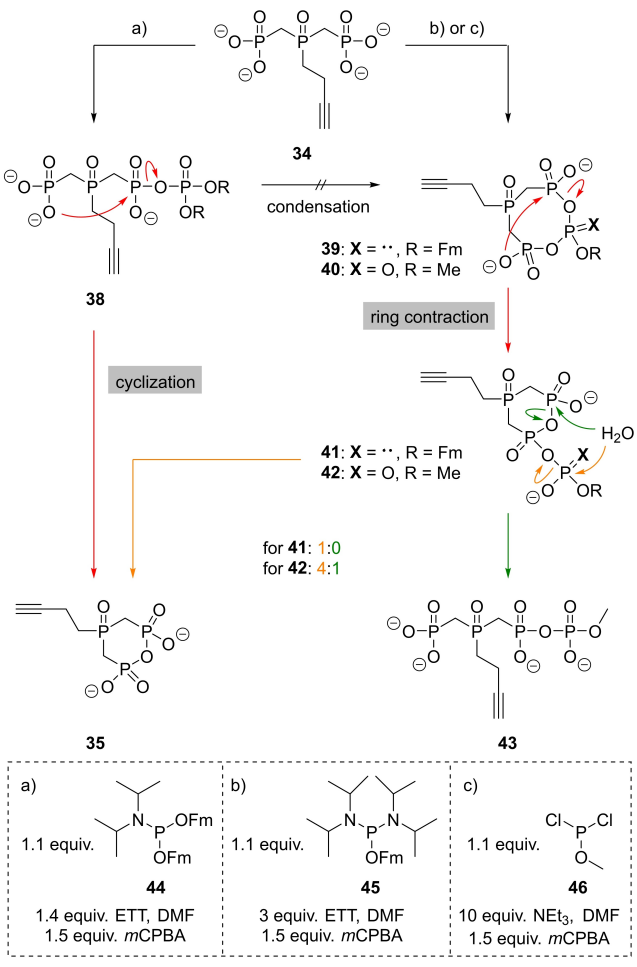


Scheme 2. Synthesis of a trimetaphosphonate pull-down probe. a) Attempt to directly modify bis(difluoromethylene)trimetaphosphate (**17**) using aryne chemistry. Identified products of the reaction are shown in the Supporting Information. b) Synthesis of a bismethylene triphosphate with arylalkynyl-modified phosphinate function. Selective cleavage of the phosphonate ethyl esters using TMSBr and spontaneous decomposition of **27**. c) Synthesis of the non-hydrolysable trimetaphosphate analogue **35** with an alkyne function attached to a phosphine oxide substructure. Crystal structure of **35** with ellipsoids drawn at 50% probability level. Functionalization of **35** using CuAAC and amide formation with sulfo-SBED (sulfo-succinimidyl-2-(6-(biotinamido)-2-(*p*-azidobenzamido)hexanoamido)ethyl-1,3′-dithiopropionate).

yield. The introduced amine moiety enabled ready modification with commercially available sulfo-SBED, containing biotin and a photoreactive phenyl azide group. Recently, this linker was successfully used by us to determine the (p)ppGpp interactome.[51] The trifunctional pull-down probe **37** was obtained in 70% yield for the last step.

Phosphoramidite chemistry with linear triphosphonate

Since larger cyclic phosphates than trimetaphosphate have also been identified in biology,[3,11,52] we intended to take advantage of the hydrolytically stable alkyne modification in **34** and **35** and increase the ring-size using phosphoramidite chemistry (Scheme 3). The reaction of **34** with fluorenylmethyl (Fm) protected phosphoramidite **44** gave the tetraphosphonate/phosphate analogue **38** only as an intermediate, which cyclized within minutes to trimetaphosphonate **35**. Therefore, we reacted **34** with phosphordiamidite **45** to directly obtain the cyclic tetrametaphosphonate product. However, after twofold coupling, the resulting intermediate **39** underwent an intramolecular nucleophilic attack of one phosphonate onto another, resulting in ring contraction and formation of **41**, reminiscent of a cyclic ultraphosphonate containing a P(III)-P(V)-anhydride (Scheme 3). Next, **41** was hydrolyzed giving again trimetaphosphonate **35** as the product. Changing to basic conditions, **34** was treated with methyl dichlorophosphite (**46**) in presence of NEt_3. After oxidation, signals at $\delta = -24$ ppm indicated successful formation of **40**. However, trimetaphosphonate **35** was also already present as judged by $^{31}P\{^1H\}$-NMR and found to be the main product after precipitation while only traces of **40** were left. Attempts to isolate **40** failed but linearized **43** – as another decomposition product of **42** – was purified and characterized (see Supporting Information).



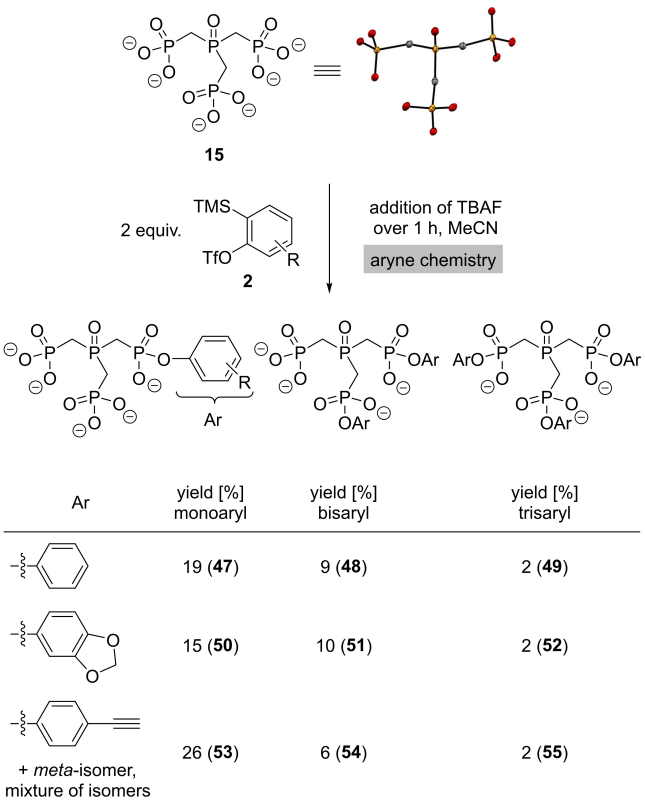
Scheme 3. Cyclization of the linear tetraphosphonate/phosphate analogue **38** and ring contraction of tetrametaphosphonate analogues **39** and **40** resulting in trimetaphosphonate **35**. For **42**, linearization to **43** was observed additionally.

In analogy to the ring-opening of cyclic ultraphosphates and functionalized trimetaphosphates, one would have expected **43** as the single hydrolysis product.[12,14,19,32,53] Also the rate of trimetaphosphate formation was unexpectedly high, suggesting that geometrical differences caused by methylene substitution of oxygen influence the reactivity. Considering the "hydrolysable" analogues, Glonek and Myers already reported slow conversion of larger metaphosphates to trimetaphosphate with intermediary formation of cyclic ultraphosphates but details of the mechanism remained elusive.[17,54] Computational results by Cummins suggest a plausible mechanism,[20] supporting the pathway shown in Scheme 3. In addition, Cummins recently showed that the $P_2O_5(pyridine)_2$ adduct does not form tetrametaphosphate with the bis(triphenylphosphine)iminium (PPN) salt of pyrophosphate but orthophosphoryl trimetaphosphate, underlining the high propensity to form trimetaphosphate rings.[27]

Ultraphosphonate aryne reaction

To study the potential interactome of ultraphosphates, the non-hydrolysable analogue **15**, called ultraphosphonate, was chosen as selection function for a pull-down probe. **15** was synthesized according to a procedure of Maier.[48,55] The corresponding TBA salt was obtained as an oil, which slowly crystallized during storage at 4 °C allowing for X-ray analysis (Scheme 4). Regarding the functionalization of **15**, we were interested to study whether an aryne reaction would result in O-arylated products as in the aryne phosphate reaction[21] or in P–C bond formation as observed for phosphinates and phosphonate esters by insertion of arynes into P–O bonds.[56]

Using Kobayashi-type *o*-silylphenyltriflate aryne precursors, we found O-arylation and mono- to trisarylated products with formation of phosphonate monoester preferred over diester formation under the applied conditions (Scheme 4). Reactions with 2 equiv. of aryne precursor typically showed ca. 25% starting material left and ca. 40% of the desired monoarylated product as indicated by $^{31}P\{^1H\}$-NMR reaction control. Around 25% were found to be bisarylated. The products were readily separated using a C18 AQ column and isolated with the yields indicated in Scheme 4. Due to the propensity of **15** to act as a nucleophile in additional arylations, but at the same time to intramolecularly condense to metaphosphonate substructures,



Ar	yield [%] monoaryl	yield [%] bisaryl	yield [%] trisaryl
	19 (**47**)	9 (**48**)	2 (**49**)
	15 (**50**)	10 (**51**)	2 (**52**)
+ *meta*-isomer, mixture of isomers	26 (**53**)	6 (**54**)	2 (**55**)

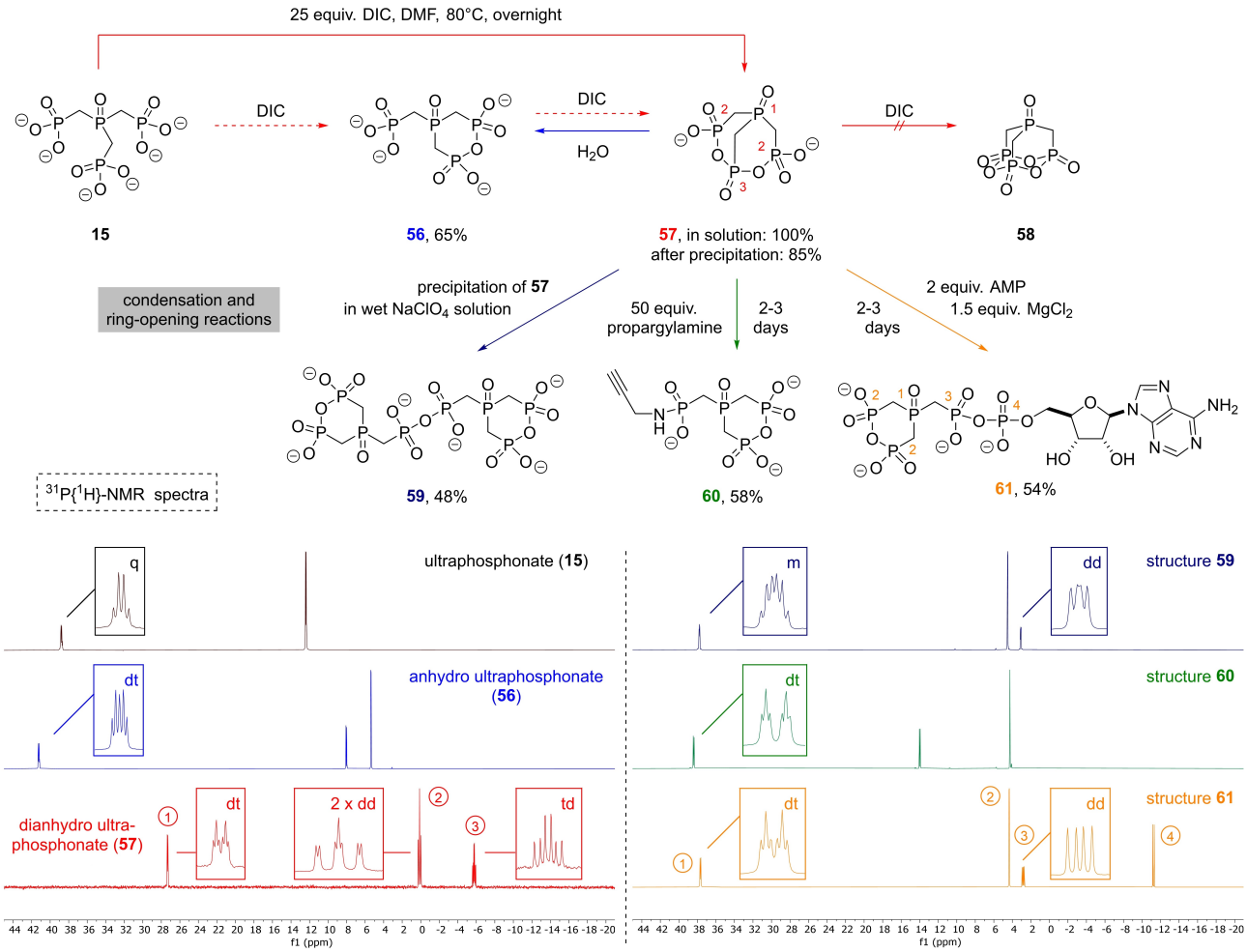
Scheme 4. Ultraphosphonate aryne reactions using Kobayashi-type aryne precursors. Crystal structure of **15** with ellipsoids drawn at 50% probability level.

these yields are acceptable. The aryne reaction with **19** results in positional isomers and a ratio of *para*- vs. *meta*-substitution of 2:1 was found for monoarylated **53**, while bis- and trisarylated **54** and **55** were isolated as more complex isomeric mixtures, which we did not separate and assign. Synthetically, the formation of regioisomers might appear disadvantageous but for the preparation and successful application of pull-down probes higher structural variety can actually be helpful.

Ultraphosphonate condensation chemistry

Since cyclic ultraphosphates are structurally closely related to non-cyclic ultra- as well as metaphosphates and show constitutional isomerism with the latter, we were interested to also synthesize and functionalize the non-hydrolysable analogue **56** (Scheme 5). Preparation of the fully dehydrated species **58** – representing an analogue of P_4O_{10} – by intramolecular dehydration of **15** using carboxylic anhydrides or acyl chlorides at elevated temperatures was reported by Maier and Kerst.[57,58] The "full" anhydride is rapidly hydrolysed to anhydride **56**, whereas further hydrolysis to non-cyclic ultraphosphonate is only achieved after 8 h at 80 °C.[57] We studied the chemistry of **15** in presence of DIC as condensing agent and found that the dianhydro product **57** is already formed prior to consumption of non-cyclic ultraphosphonate **15**. 25 equiv. DIC were sufficient for full turnover to **57** at 80 °C overnight. The $^{31}P\{^1H\}$-NMR spectrum of **57** in DMF-d_7 is shown in red in Scheme 5. Notably, we did not observe formation of the adamantane structure **58** even at higher excess of DIC. While no rational synthesis of dianhydro ultraphosphonate (**57**) was known yet, our finding is in accordance with reports on carbodiimide-mediated condensation reactions of phosphoric acid, linear and cyclic polyphosphates as well as mixtures of these, leading essentially to only 1,5-μ-oxo-tetrametaphosphoric acid.[59] Dianhydro ultraphosphonate (**57**) was precipitated as its TBA salt with minor decomposition (<2%) using Et_2O. It contained residual urea byproduct from the condensing agent. Remarkably, precipitated **57** was not only soluble in DMF, but also in acetone and chloroform and showed no decay in further analyses of the solutions several days later.

The yield for **57** was determined by addition of a defined volume of aqueous phosphonoacetic acid solution and integration against the hydrolysis product anhydro ultraphosphonate (**56**). Using this method, we determined a yield of 85% for precipitated **57**. The latter could be stored at −20 °C with only minimal decomposition detected after two weeks. Using $NaClO_4$ solution in acetone for precipitation of **57** gives the corresponding sodium salt, which is only soluble in water. $^{31}P\{^1H\}$-NMR spectra immediately after dissolution of **57** sodium salt in water showed around 50% of **57**, which was consumed after 90 min. However, **56** was not the single hydrolysis product but dimerized anhydro ultraphosphonate **59** – as the product of a nucleophilic attack of anhydro ultraphosphonate **56** on dianhydro ultraphosphonate **57** – was formed as well with a ratio of 4:1 **56** vs. **59** (Scheme 5). The rate of **59** formation was increased to ca. one third using a wet $NaClO_4$ solution in acetone (5% water) for precipitation. After SAX purification, **59** was thus obtained in 48% yield. The $^{31}P\{^1H\}$-NMR spectrum is shown in dark blue in Scheme 5. To establish a protocol for the synthesis of anhydro ultraphosphonate **56** starting form **57**, we attempted to avoid the formation of **59** but could only reduce its amount to 5–10% by adding an acetone solution of **57** dropwise to water. Following this procedure, we obtained 65% of **56** after SAX-purification (Scheme 5, in blue). In addition to water, propargylamine and AMP were found to be appropriate nucleophiles for ring-opening of **57**. Of note, trials adding propargylamine together with the condensing agent and prior to completed formation of **57** suggest that ultraphosphonate first undergoes intramolecular dehydration followed by nucleophilic ring-opening rather than direct condensation with the amine. In accordance, we found no propargylamino-modified, non-cyclic ultraphosphonate in these reactions. Ring-opening of **57** to **60** was finished after two to three days when 50 equiv. propargylamine were used and the product **60** isolated in 58% yield (Scheme 5, in green). Strikingly, **57** only underwent ring-opening at the Q^2 phosphorus. This is in contrast to computational results for the "hydrolysable" analogue 1,5-μ-oxo-tetrametaphosphoric acid revealing a ca. 5 kcal/mol higher thermodynamic barrier for nucleophilic attack of a Q^2 vs. a Q^3 phosphorus and tetrametaphosphate structures as the kinetic products.[20] Formation of a modified tetrametaphosphonate by nucleophilic attack of the Q^3 phosphorus in **57** is however not possible due to the non-hydrolysable CH_2 bridge between the



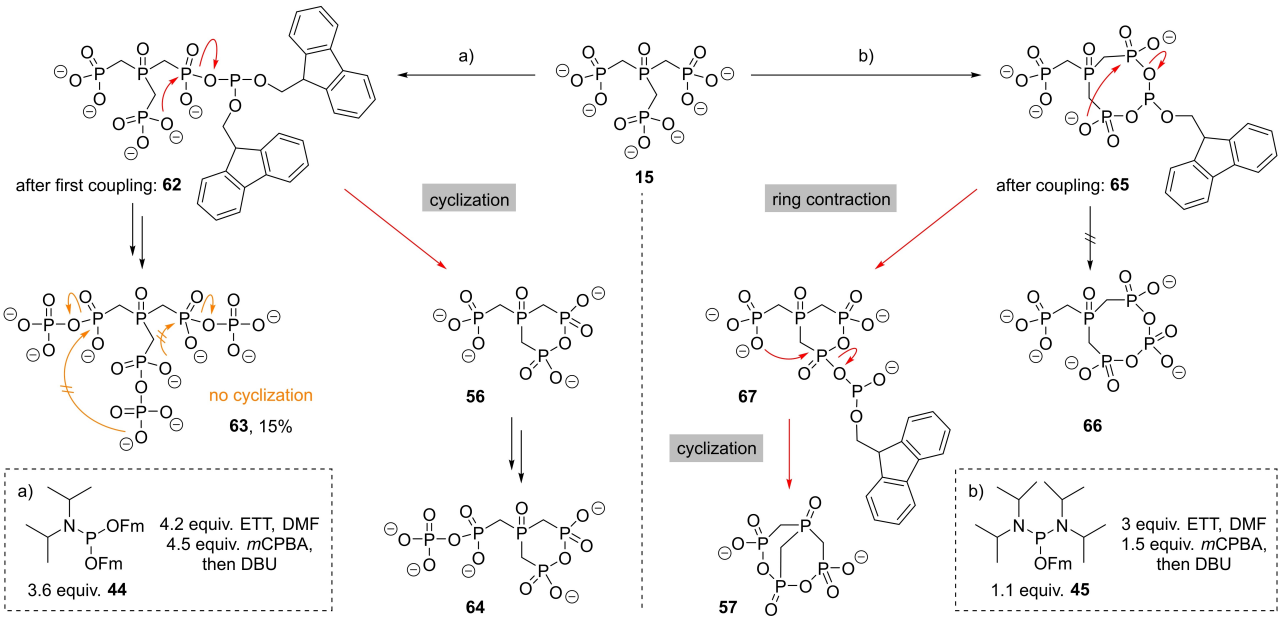
Scheme 5. Condensation of ultraphosphonate (**15**) and ring-opening of dianhydro ultraphosphonate (**57**) with different nucleophiles. $^{31}P\{^{1}H\}$-NMR were measured in D_2O expect for **57**, which was measured in DMF-d_7. Signals without expanded view are doublets.

two Q^3 phosphorus atoms. The enforced reactivity may be energetically unfavourable and could rationalize the required large excess of nucleophile. Further ring-opening of **60** to non-cyclic bispropargylamino ultraphosphonate was not observed even with 100 equiv. propargylamine or at 80 °C. Taking AMP TBA salt as nucleophile gave only traces of **61**, but addition of 1.5 equiv. $MgCl_2$ promoted the ring-opening in accordance with reports on similar reactions with cyclic ultraphosphates.[33] With Mg^{2+} as additive, 2 equiv. AMP were sufficient to consume **57** within two to three days. After SAX-purification, the product **61** was obtained with 54% yield (Scheme 5, in orange).

Phosphoramidite chemistry with ultraphosphonate

Structurally related to **61**, unmodified phospho ultraphosphonate (**64**) was observed as the main product, when ultraphosphonate(**15**) was reacted with 3.6 equiv. Fm-protected phosphoramidite **44** (Scheme 6). While **64** and **56** – products of cyclization reactions – were only obtained as mixture, trisphospho ultraphosphonate **63** could be isolated with 15% yield. Interestingly, **63** showed no cyclization reaction anymore. As already reported for ultraphosphonate,[60] such structures can act as potent chelating agents and the ability to complex different metals can be expected to be further increased for **63**. Considering trisphospho ultraphosphonate (**63**) as model structure for ultraphosphates with elongated phosphate chains, this so far uncharacterized structural motif may have sufficient stability to become accessible as well.[39]

As we observed a high tendency to form trimetaphosphonate (sub)structures (Scheme 3), we were interested to see whether a cyclic ultraphosphonate, containing a tetrametaphosphonate subunit, can be synthesized. We thus treated ultraphosphonate with 1.1 equiv. of phosphordiamidite **45** and detected dianhydro ultraphosphonate (**57**) and Fm-phosphonate after the coupling step (Scheme 6). Formation of **57** requires two condensation steps suggesting that a tetraphosphonate-phosphite **65** was successfully formed as intermediate. We propose that this mixed P(III)-P(V) anhydride undergoes intramolecular nucleophilic attack of one phosphonate of the ring onto another resulting in ring contracted **67** with a non-cyclic P(III)-P(V)-anhydride. The resulting phosphite diester (or isomerized phosphonate) then acts as leaving group for a



Scheme 6. Reactions of ultraphosphonate (**15**) with phosphoramidite **44** and -diamidite **45**. The transient products **62**, **65** and **67** underwent cyclization reactions respectively ring contraction.

nucleophilic reaction of the non-cyclic phosphonate (Scheme 6).

Pull-down probes of linear and branched condensed phosph(on)ates

For the construction of a pull-down probe, anhydro ultraphosphonate (**56**) was reacted with alkyne-tagged aryne precursor **19** to enable further modifications. The product **68** (Scheme 7) was obtained in 14% yield with a regioisomeric ratio of 2:1 (*para* vs. *meta*). As observed for metaphosphonate **18** as well (Scheme 2a), fluoride acted as nucleophile to partially open the ring-structure, explaining the low yield of the reaction. No further optimisation was conducted at this point.

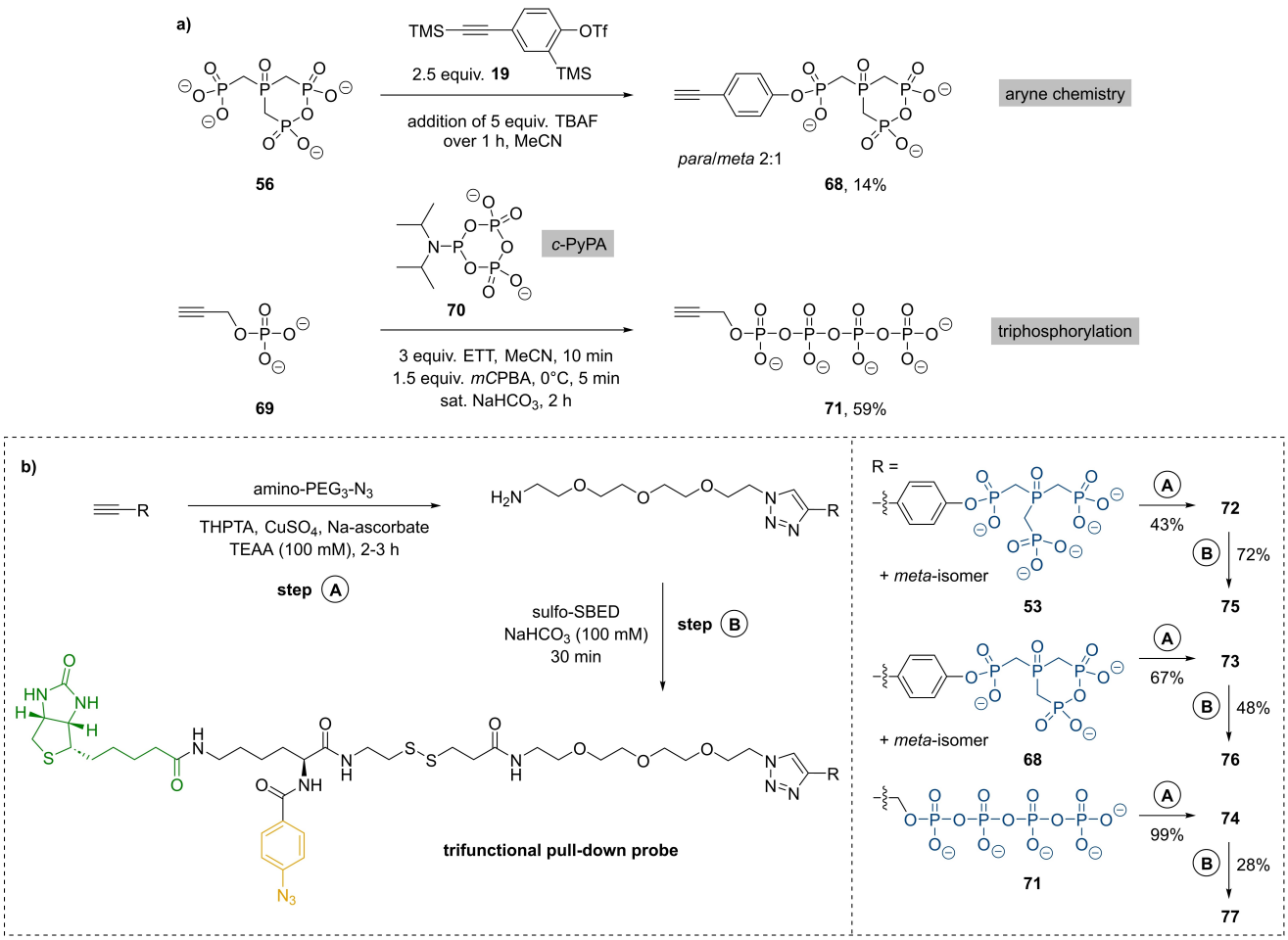
Since ultraphosphate and linear tetraphosphate are constitutional isomers, the latter is an important negative control to determine, whether proteins were bound in a pull-down experiment due to a constitutional preference or just because of similar ionic interactions. The sufficient stability of tetraphosphate[61] rendered the synthesis of a non-hydrolysable analogue unnecessary. Propargyl phosphate (**69**) was triphosphorylated using cyclic pyrophosphoryl phosphoramidite (70, *c*-PyPA)[33,53] giving 59% 71.

Analogous to the synthesis of the trimetaphosphonate pull-down probe (Scheme 2c), alkyne-tagged **53**, **68** and **71** were reacted with amino-PEG_3-azide in CuAAC click reactions with 43%, 67% and 99% yield, respectively. Since ultraphosphonate is a strong complexing agent,[60] water-soluble tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was added as ligand to keep copper available for the click-reaction. Amine-functionalized **72**, **73** and **74** were then transformed into the pull-down probes by incubation with sulfo-SBED in $NaHCO_3$ buffer (100 mM) with 72%, 48% and 28% yield, respectively.

Pull-down experiments with yeast cell lysates

Photoaffinity capture compounds are typically used in combination with proteomics, which enables identification of interactors from complex mixtures such as cell lysates with high specificity. Such chemical proteomics approaches were applied to a wide range of substrates – for example (p)ppGpp or cyclic di-GMP – and organisms.[51,62–65] The methodology described in those references is now adapted herein to pull down proteins binding to different condensed (cyclic or branched) phosphate analogues to delineate the elusive interactome of non-linear polyphosphates. The pull-down experiments were conducted with yeast cell extracts, since inorganic polyphosphate is highly abundant in yeast[66] and the enzymology is well described.[10,67] Moreover, early studies reported the presence and metabolism of trimetaphosphate in yeast extracts albeit uncertainties remained whether these findings are analytical artefacts.[3–7,61] To study interactomes related to phosphate supply, yeast may be cultured under phosphate depletion, resupply conditions or in phosphate-rich media. We decided to prepare lysates from BJ3505 WT with no phosphate present in the culture medium. Future studies could be designed to investigate the interactome under alternative conditions.

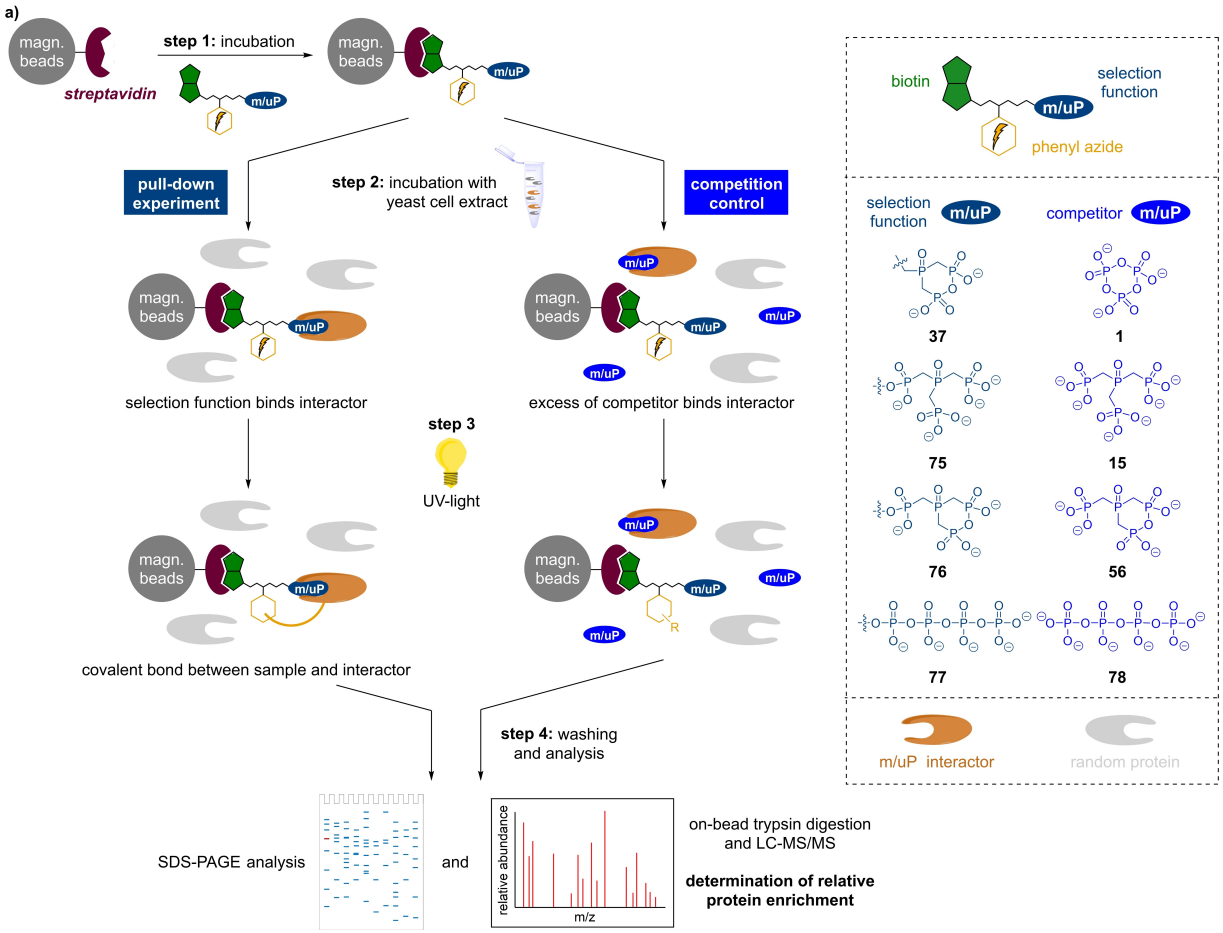
The experimental procedure of the photoaffinity pull-down experiments is shown in Scheme 8a: each capture compound was initially incubated with streptavidin-coated magnetic beads to ensure high loading (step 1). This procedure was developed using SDS-PAGE control experiments that showed in general higher protein enrichment as the reverse process: incubating

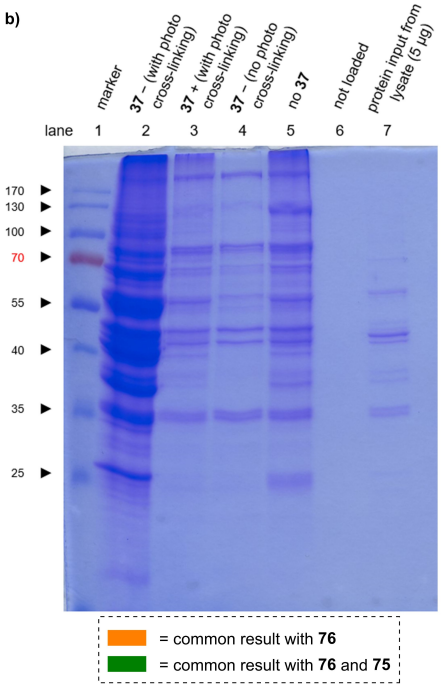


Scheme 7. Synthesis of trifunctional pull-down probes containing ultraphosphonate (**75**), anhydro ultraphosphonate (**76**) and linear tetraphosphate (**77**) as selection function. a) Synthesis of alkyne-modified anhydro ultraphosphonate **68** and propargyl tetraphosphate (**71**). b) Development of alkyne-modified condensed phosph(on)ates **53**, **68** and **71** into pull-down probes *via* click-reaction and amide coupling.

the probe first with the lysate and then with the beads. Therefore, in the second step, the beads were incubated with the yeast cell lysate either in absence (pull-down experiment) or presence of ca. 300-fold excess of competitor (competition control). The competition control should reduce false-positive hits as the high excess of the unmodified structure of interest (competitor) should preferentially occupy the active site of interactors suppressing their interaction with the capture compound while unspecific interactions are not prevented. False-positive hits of the pull-down experiment can thus be reduced by comparison with the results obtained in absence of the competitor. The mixture was irradiated with UV light in step 3 to induce photo cross-linking by transformation of the phenyl azide into a reactive nitrene. The latter can undergo C–H insertion forming covalent bonds with other molecules in direct vicinity – usually an interactor of the selection function. This stable linkage then enables stringent washing of the beads, reducing false-positive hits (step 4). Each experiment was performed in duplicate and one sample was used for trypsination and LC-MS/MS analysis while the other sample was subjected to SDS-PAGE analysis to validate the procedure by comparison of the number and intensity of obtained bands. Without competitor, distinct bands should show increased intensity indicating enrichment of putative interactors whereas the competition control reveals unselectively bound proteins.

SDS-PAGE analyses of the initial experiments indicated strong unspecific binding since the pull-down experiment and competition control only showed slight differences and similar bands were obtained for all capture compounds. Importantly, the negative control containing no capture compound showed the same bands as the pull-down experiment pointing towards a loss of the selection function. We assumed that high concentrations of reduced glutathione[68] caused cleavage of the disulfide bridge in the capture compounds. Reductive cleavage of the commercial BED linker was undesired in our experiments. We therefore performed further pull-down experiments under oxidative conditions by incubation of the lysate with 0.1% H_2O_2, which should convert glutathione to its oxidized form and thus ensure stability of the capture compound. Since only the lysate is treated with H_2O_2, the proteome remains unaffected by upregulation or repression of gene transcription associated with oxidative stress response.[69] To test our hypothesis, we performed initial pull-down experiments under oxidative conditions with **37**. Scheme 8b shows





c)

entry	gene	protein	log_2 (enrichment)
1	*MSE1*	glutamate-tRNA ligase, mitochondrial	5.5
2	*ECT1*	ethanolamine-phosphate cytidylyltransferase	5.0
3	*GPM3*	phosphoglycerate mutase 3	4.8
4	*RIB3*	3,4-dihydroxy-2-butanone-4-phosphate synthase	4.5
5	*MSK1*	lysine-tRNA ligase, mitochondrial	3.6
6	*QRI1*	UDP-N-acetylglucosamine pyrophosphorylase	3.5
7	*HXK1*	hexokinase 1	3.5
8	*GUK1*	guanylate kinase	3.5
9	*ERG8*	phosphomevalonate kinase	3.2
10	*HXK2*	hexokinase 2	3.0
11	*TPK3*	cAMP-dependent protein kinase type 3	3.0
12	*CKI1*	choline kinase	2.9
13	*ARG82*	inositol polyphosphate multikinase	2.8
14	*TRL1*	tRNA ligase	2.7
15	*SLM5*	asparagine-tRNA ligase, mitochondrial	2.7
16	*APT2*	adenine phosphoribosyltransferase 2	2.6
17	*CAB2*	phosphopantothenate-cysteine ligase CAB2	2.5
18	*APT1*	adenine phosphoribosyltransferase 1	2.4
19	*FUR1*	uracil phosphoribosyltransferase	2.3
20	*CAB4*	phosphopantetheine adenylyltransferase	1.9
21	*MET16*	phosphoadenosine phosphosulfate reductase	1.9
22	*PPX1*	exopolyphosphatase	1.8
23	*APA1*	Ap4A phosphorylase; ADP-sulfurylase	1.7
24	*ARG1*	argininosuccinate synthase	1.6
25	*ADK1*	adenylate kinase	1.6

Scheme 8. Pull-down experiments with capture compounds **37**, **75**–**77** and yeast cell extracts. a) Experimental procedure: step 1: incubation of streptavidin magnetic beads with a capture compound. Step 2: incubation of the beads with yeast cell extracts without (pull-down experiment) or with competitor (competition control). Step 3: induction of photo cross-linking by irradiation with UV light. Step 4: washing of the beads. Analysis by SDS-PAGE respectively on-bead trypsin digestion of captured proteins and LC-MS/MS. b) SDS-PAGE of pull-down and control experiments with capture compound **37** under oxidative conditions (0.1 % H_2O_2). − = no competitor; + = with competitor. Lane 1 = marker; lane 2 = pull-down experiment; lane 3 = competition control; lane 4 = cross-linking control; lane 5 = beads control; lane 6 = not loaded; lane 7 = protein input from lysate (5 µg). c) Exemplary hit-list for capture compound **37**. Proteins, which were also captured for **76**, are marked in orange. Common hits for **37**, **76** and **75** are marked in green.

the result for the corresponding SDS-PAGE analysis. Additional bands for the pull-down experiment (lane 2) compared with the negative control containing no **37** (lane 5) now suggested retention of the selection function. The competition control (lane 3) showed less bands indicating selective protein binding and strong intensity differences point towards successful enrichment of proteins. The overall lower intensity of the bands in lane 4 – corresponding to a sample, which was not UV-irradiated – furthermore suggest successful photo cross-linking. We thus adapted the optimized procedure to the further capture compounds **75**–**77**. SDS-PAGE analysis indicated selective binding of proteins albeit differences between number and intensity of bands were weak (Figure S1). Similar results for the different capture compounds may arise from their structural relationship but could also indicate general binding of proteins to densely charged structures. Since interacting proteins may only show weak bands, their determination by PAGE analysis is hampered impeding further evaluation by this analytical method. Therefore, we analysed the samples by on-bead trypsin digestion and LC-MS/MS analysis. The data are available *via* ProteomeXchange with the dataset identifier PXD043919. We chose $\log_2(\text{enrichment}) > 1.5$, corresponding to a ca. 3-fold increase of protein in the pull-down compared to the competition control experiment, and razor unique peptides ≥ 3 as threshold for considering a protein as hit. 258 proteins were identified for **37** matching this criteria, 35 for **75**, 115 for **76** and 11 for **77**. The proteins are listed in the Supporting Information. Exemplarily, Scheme 8c shows selected hits for capture compound **37**. Results, which were obtained for both **37** and **76** are marked in orange in Scheme 8c; common results for **37**, **76** and **75** are indicated in green. Due to the close structural relation, overlapping hits were expected and support the quality of the data. In line, the results for linear tetraphosphate differed markedly. However, it is unclear whether this can be assigned to structural preferences or if the low number of captured proteins is a result of partial degradation of the "hydrolysable" capture compound **77** in the cell extract. We did not use phosphatase inhibitors to avoid blocking interaction sites our probes could bind to.

Although methylene-substitution is generally accepted as a good bioisosteric replacement for phosphoanhydride bonds,[25] different bond lengths and angles – as determined from crystallographic data for **35** as well (Scheme 2c) –, reversed polarity of the CH_2-modification[24] or lacking hydrogen bonds may lower binding affinities. This is exemplified by a report of Blackburn that yeast exopolyphosphatase (PPX) only bound adenosine tetraphosphate with native P–O–P bridges.[70] Yet, in our example, PPX was captured by pull-down probe **37** that contains methylene bridges. The susceptibility of metaphosphate esters to linearization and the sensitivity of ultraphosphates towards several conditions obviously require stabilization of anhydride bonds for development into affinity reagents. The pull-down data provided here serve as valuable starting point for further investigations, while additional efforts should be made to establish new methods, compatible with the reactivity and properties of non-linear condensed phosphates.

Conclusions

In the present paper, we disclosed syntheses of capture compounds containing non-hydrolysable analogues of cyclic and branched condensed phosphates as selection function and applied them in photoaffinity pull-down experiments with yeast cell extracts. Along the way, we developed new phosphonate chemistry and studied the transformations that occur in these systems.

We found that methylene-bridged trimetaphosphate analogues are poor substrates for aryne chemistry due to several side reactions and that arylesters at the phosphinate function of a bismethylene trisphosphate are prone to hydrolysis. Therefore, an alkyne tagged bis(chloromethyl)phosphine oxide was prepared and further reacted to the bismethylene triphosphate analogue using an Arbuzov reaction. After condensation to the corresponding trimetaphosphonate, we detected only little hydrolytic ring-opening after several days in solution allowing the development into pull-down probes. Efforts to prepare a tetrametaphosphonate sample revealed a strong preference to form trimetaphosphonate structures by either cyclization or ring contraction. Similar results were obtained for ultraphosphonate, which only formed a transient tetrametaphosphonate subunit and underwent fast consecutive reactions to form dianhydro ultraphosphonate. The latter was selectively accessible using DIC for condensation of ultraphosphonate and could be ring-opened by water to yield anhydro ultraphosphonate or amine and phosphate nucleophiles. Ultraphosphonate and anhydro ultraphosphonate were successfully functionalized using aryne chemistry and further transformed into pull-down probes.

Pull-down experiments with yeast cell extracts were performed under oxidative conditions. With the chemical proteomics approach, we could identify numerous putative interactors of non-linear polyphosphates providing a first entry into their elusive interactomes. Our data serve as a starting point for further investigations giving prospects to not only identify cyclic and branched phosphates as enzyme substrates but also their non-hydrolysable analogues as potential inhibitors. Enzymatic reactions including four phosphates may be of special interest as they could potentially proceed *via* a phosphate walk-like reaction[27,39] – a so far unconsidered reactivity, which would involve branched condensed phosphates as intermediates. Concerning metaphosphates, the formation of aminoacyladenylate analogues in presence of trimetaphosphate has already been reported under prebiotically plausible conditions.[71] This supports the idea that trimetaphosphate could indeed act as substrate for tRNA ligases, of which we identified several in this pull-down (Scheme 8c and Table S1). While these examples only illustrate considerations about the plausibility of the obtained data, the latter may aid to comprehensively study the potential biology of non-linear polyphosphates. Based on recent reports on the presence of metaphosphates in organisms and the interaction of synthetic ultraphosphates with alkaline phosphatase, we are confident that a rich biology of cyclic and branched condensed phosphates awaits its discovery.

Experimental Section

General experimental remarks, detailed synthetic procedures and analyses by NMR and mass spectrometry are described in the Supporting Information.

Arbuzov reactions with bis(chloromethyl) phosphinates or phosphine oxides: A solution of a bis(chloromethyl) phosphinate or phosphine oxide (1.0 equiv.) in triethyl phosphite (12 to 21 equiv.) was stirred at 155 °C overnight. The reaction mixture was subjected to silica gel and purified using a PuriFlash Column (SI HP 30 μm, pure AcOEt to AcOEt/MeOH 4:1).

General procedure for dealkylation using TMSBr: A solution of a triphosphonate ethyl ester (1.0 equiv.) in CH_2Cl_2 (100 mM) was treated with a solution of TMSBr (10 equiv.) and pyridine (25.0 equiv.) in CH_2Cl_2 (ca. 3 M referring to pyridine) at 0 °C for 5 h. The mixture was allowed to slowly reach room temp. and further stirred overnight. Water was added, the aqueous layer separated and evaporated to dryness. The residue was dissolved in water and the pH adjusted to 8.5 using NaOH solution (1 M). It was purified by AIEX chromatography (Q Sepharose® Fast Flow, increasing concentrations of $NaClO_4$). The product was precipitated using ice-cooled acetone (product fraction/acetone: 1:9 v/v), washed with acetone twice and dried *in vacuo*.

General procedure for aryne reactions: Ultraphosphonate TBA salt (1.0 equiv.) was dissolved in MeCN (ca. 60–80 mM) and 2-(trimethylsilyl)phenyl trifluoromethanesulfonate or a respective aryne precursor (2.0 equiv.) was added. TBAF (1 M in THF, 2.0 equiv.) was added over 1 h using a syringe pump (needle inside solution). It was precipitated using an ice-cooled solution of $NaClO_4$ in acetone (0.5 M), the suspension centrifuged and the pellet washed with acetone twice. After drying *in vacuo*, the crude product was purified using a PuriFlash Column (30 μ C18 AQ, MeCN gradient (0–35 %), 10 % TEAA (100 mM, pH 7.0)). Lyophilization afforded the product as triethylammonium salt.

General procedure for click reactions with amino-PEG_3-azide: An alkyne-modified condensed phosph(on)ate (1.0 equiv.) was dissolved in TEAA (100 mM, pH 7.0; reaching ca. a 5 mM phosph(on)ate solution) and degassed by bubbling a stream of argon through the solution for 10 min. Sodium ascorbate (10.0 equiv.) and $CuSO_4 \cdot 5\ H_2O$ (10 mg/ml in water, 1.0 equiv.) were added. Amino-PEG_3-azide (1.2 equiv.) was added and stirred for 3 h. It was precipitated using an ice-cooled solution of $NaClO_4$ in acetone (0.5 M), washed with acetone twice and dried *in vacuo*. The crude product was purified using a PuriFlash Column (15 μ C18 AQ, MeCN gradient (0–35 %), 10 % TEAA (100 mM, pH 7.0)). Lyophilization afforded the product as triethylammonium salt.

General procedure for amide formation with sulfo-SBED: A PEG_3-amino-modified condensed phosph(on)ate (10 μg/μl in $NaHCO_3$ buffer (200 mM), 1.0 equiv.) was mixed with a solution of sulfo-SBED (10 μg/μl in water, 1.4 equiv.) and incubated for 30 min. The reaction mixture was directly subjected to purification using a PuriFlash Column (15 μ C18 AQ, MeCN gradient (0–50 %), 10 % TEAA (100 mM, pH 7.0)). Lyophilization afforded the product as triethylammonium salt

Condensation of ultraphosphonate (15) and ring-opening of dianhydro ultraphosphonate (57): DIC (25.0 equiv.) was added to a solution of ultraphosphonate TBA salt in DMF (ca. 80 mM, 1.0 equiv.) and stirred at 80 °C overnight. It was precipitated using Et_2O, washed with Et_2O twice and dried *in vacuo*. Dianhydro ultraphosphonate (ca. 85 μmol) was dissolved in DMF (ca. 20–80 mM). The nucleophile (up to 50 equiv. depending on specific nucleophile) – and in case of a phosphate nucleophile $MgCl_2$ (1.5 equiv.) – were added and the mixture stirred for 2–3 days. It was precipitated using an ice-cooled solution of $NaClO_4$ in acetone (0.5 M) and washed with acetone twice. After drying *in vacuo*, the crude product was purified by AIEX chromatography (Q Sepharose® Fast Flow, increasing concentrations of $NaClO_4$). The product was precipitated using ice-cooled acetone (product fraction/acetone: 1:9 v/v), washed with acetone twice and dried *in vacuo*.

Crystallographic data: Deposition number(s) 2127320 (for **35**), 2100905 (for **15**), and 2114836 (for **S11**) contain(s) the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures..

Pull-down experiments: The procedure was adapted from Jenal.[62,64,65] Cell extracts from yeast BJ3505 WT were grown with no P_i present in the medium. Experiments performed in duplicates. Details on the used buffer solutions are described in the Supporting Information.

Lysate preparation

1. Grow *S. cerevisiae* in SC medium to the desired OD (OD_{600nm} ca. 1.5).
2. Pellet 750 OD by centrifugation for 2 min at 4,000 rpm.
3. Wash pellet in 15 ml lysis buffer and centrifuge. Resuspend pellet in 10 ml lysis buffer with protease inhibitor and DNase I.
4. Lyse the cells through a French pressure cell, at 1.9 kbar.
5. Incubate lysate with 0.1 % H_2O_2 (40 μl 30 % H_2O_2 in 12 ml lysate) for 10 min on a rotating wheel.
6. Ultra-centrifuge the cell lysate at 100,000×*g* (37.000 rpm, TI70 fixed angle rotor) for 1 hr at 4 °C.
7. Transfer 9 ml of the supernatant; measure protein concentration using NanoDrop.
8. Prepare input sample for SDS-PAGE analysis: 50 μl protein extract + 50 μl 2×Lämmli/100 mM DTT. Heat 5 min to 95 °C.

Capture solutions

Prepare solutions of protein (ca. 18 mg protein) with 300 μl 5×capture buffer, with and without 15 μl competitor (40 mM stock). Dilute with water to 1403 μl. Incubate at 4 °C for 30 min on a rotating wheel.

Per sample: wash 90 μl streptavidin magnetic beads (10 mg particles per ml; binding capacity per mg: 1800 pmol free biotin) with 1×capture buffer. Resuspend in 90 μl 1×capture buffer. Add 3 μl 5×wash buffer and 4.5 μl capture compound (400 μM stock). Incubate at 4 °C for 30 min under slight shaking.

Mix and bind

Add the protein/competitor mixture to the beads/capture compound suspension.

Incubate at 4 °C for 2 h on a rotating wheel. Centrifuge briefly, resuspend and transfer samples to a 12-well plate.

Cross-link

Cross link in 12-well plate on ice under xenon light for 2 min. Transfer sample to Eppendorf tube on magnetic rack. Collect the supernatant. Rinse the well with 1 ml 1×washing buffer and add solution to the beads.

Washing Steps

Wash the beads in Eppendorf tubes on a magnetic rack

1. Wash 1× with 1 ml 1×washing buffer and 2× with 0.5 ml 1×washing buffer.
2. Wash 1× with 750 μl H_2O.
3. Wash 3× with 750 μl 80% MeCN.
4. Wash 2× with 1 ml 1×washing buffer with Triton X-100. Centrifuge and remove washing buffer.
5. Suspend in 620 μl 1×washing buffer with Triton X-100.

Samples for SDS-PAGE analysis

6. Transfer 200 μl into a new Eppendorf tube.
7. Wash with 750 μl H_2O, centrifuge and remove supernatant.
8. Resuspend the beads in 50 μl 2×Laemmli buffer/100 mM DTT and heat to 95 °C for 5 min.
9. Dilute input sample to ca. 200 ng/μl (load 25 μl corresponding to 5 μg protein on gel).
10. Reheat all samples at 95 °C and load the samples on a 12.5% denaturing SDS-PAGE gel.
11. Use coomassie staining protocol for visualization of the bands.

Samples for LC-MS/MS analysis

6′. Transfer 400 μl into a new Eppendorf tube.
7′. Wash 3× with 50 mM Tris-HCl buffer pH 7.5. Centrifuge and remove all buffer. Store at −20 °C until measurement.

LC-MS/MS and data processing: Details on LC-MS/MS and data processing are described in the Supporting Information. Mass spectrometry proteomics data are available *via* ProteomeXchange with the dataset identifier PXD043919.

Supporting Information

The authors have cited additional references within the Supporting Information.[72]

Acknowledgements

We thank Dr. Manfred Keller from MagRes of the University of Freiburg for kind support and significant amount of time for NMR spectroscopy. We thank Christoph Warth for HRMS measurements and Dr. Burkhard Butschke and Boumahdi Benkmil for support in X-ray crystallography. For support in mass spectrometry-based proteomics work, we thank the Protein Analysis Facility of the Faculty of Biology and Medicine, University of Lausanne. Moreover, we acknowledge insightful comments from Dr. Manfredo Quadroni and Noah Hans Mayer. This study was supported by the VW Foundation (Experiment! AZ92270). The authors gratefully acknowledge financial support from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy (EXC-2193/1-390951807) and a scholarship grant from Cusanuswerk. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in ProteomeXchange at http://www.ebi.ac.uk/pride, reference number 43919.

Keywords: chemical proteomics · metaphosphate · non-hydrolysable analogues · ultraphosphate · yeast

[1] J. J. Christ, S. Willbold, L. M. Blank, *Anal. Chem.* **2020**, *92*, 4167.
[2] L. Xie, U. Jakob, *J. Biol. Chem.* **2019**, *294*, 2180.
[3] V. S. Mandala, D. M. Loh, S. M. Shepard, M. B. Geeson, I. V. Sergeyev, D. G. Nocera, C. C. Cummins, M. Hong, *J. Am. Chem. Soc.* **2020**, *142*, 18407.
[4] J. E. G. Dirheimer, *Bull. Soc. Chim. Biol.* **1957**, *39*, 89.
[5] F. M. Harold, *Bacteriol. Rev.* **1966**, *30*, 772.
[6] G. W. Rafter, *Arch. Biochem. Biophys.* **1959**, *81*, 238.
[7] S. R. Kornberg, *J. Biol. Chem.* **1956**, *218*, 23.
[8] a) A. Kornberg, *J. Bacteriol.* **1995**, *177*, 491; b) M. R. W. Brown, A. Kornberg, *Trends Biochem. Sci.* **2008**, *33*, 284; c) T. V. Kulakovskaya, V. M. Vagabov, I. S. Kulaev, *Process Biochem.* **2012**, *47*, 1.
[9] a) N. Neville, N. Roberge, Z. Jia, *Int. J. Mol. Sci.* **2022**, *23*; b) H. Zhang, M. R. Gómez-García, X. Shi, N. N. Rao, A. Kornberg, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16486.
[10] S. Austin, A. Mayer, *Front. Microbiol.* **2020**, *11*.
[11] T. Glonek, *Origins Life Evol. Biospheres* **2021**, *51*, 1.
[12] S. M. Shepard, H. J. Jessen, C. C. Cummins, *J. Am. Chem. Soc.* **2022**, 7517.
[13] D. Bezold, T. Dürr, J. Singh, H. J. Jessen, *Chem. Eur. J.* **2020**, *26*, 2298.
[14] S. Mohamady, S. D. Taylor, *Org. Lett.* **2013**, *15*, 2612.
[15] S. Mohamady, S. D. Taylor, *Org. Lett.* **2016**, *18*, 580.
[16] S. M. Shepard, C. C. Cummins, *J. Am. Chem. Soc.* **2019**, *141*, 1852.
[17] T. Glonek, J. R. Van Wazer, T. C. Myers, *Phosphorus Sulfur* **1977**, *3*, 137.
[18] Y. Jiang, K. Chakarawet, A. L. Kohout, M. Nava, N. Marino, C. C. Cummins, *J. Am. Chem. Soc.* **2014**, *136*, 11894.
[19] S. M. Shepard, I. W. Windsor, R. T. Raines, C. C. Cummins, *J. Am. Chem. Soc.* **2019**, *141*, 18400.
[20] S. M. Shepard, H. Kim, Q. X. Bang, N. Alhokbany, C. C. Cummins, *J. Am. Chem. Soc.* **2021**, *143*, 463.
[21] T. M. Haas, S. Wiesler, T. Dürr-Mayer, A. Ripp, P. Fouka, D. Qiu, H. J. Jessen, *Angew. Chem. Int. Ed.* **2022**, *61*, e202113231.
[22] a) G. Weill, M. Klein, M. Calvin, *Nature* **1963**, *200*, 1005; b) H. G. Khorana, *J. Am. Chem. Soc.* **1954**, *76*, 3517; c) A. R. Kore, A. Senthilvelan, B. Srinivasan, M. Shanmugasundaram, *Nucleosides Nucleotides Nucleic Acids* **2013**, *32*, 411.
[23] a) R. Engel, *Chem. Rev.* **1977**, *77*, 349; b) S. B. Engelsma, N. J. Meeuwenoord, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov, *Angew. Chem. Int. Ed.* **2017**, *56*, 2955; c) T. G. Upton, B. A. Kashemirov, C. E. McKenna, M. F. Goodman, G. K. S. Prakash, R. Kultyshev, V. K. Batra, D. D. Shock, L. C. Pedersen, W. A. Beard, S. H. Wilson, *Org. Lett.* **2009**, *11*, 1883.
[24] G. K. Surya Prakash, M. Zibinsky, T. G. Upton, B. A. Kashemirov, C. E. McKenna, K. Oertell, M. F. Goodman, V. K. Batra, L. C. Pedersen, W. A. Beard, D. D. Shock, S. H. Wilson, G. A. Olah, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 15693.
[25] M. Wu, B. E. Dul, A. J. Trevisan, D. Fiedler, *Chem. Sci.* **2013**, *4*, 405.
[26] a) D. Furkert, S. Hostachy, M. Nadler-Holly, D. Fiedler, *Cell Chem. Biol.* **2020**, *27*, 1; b) M. Wu, L. S. Chong, D. H. Perlman, A. C. Resnick, D. Fiedler, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E6757.

[27] K. Qian, S. M. Shepard, T. Xin, G. Park, C. C. Cummins, *J. Am. Chem. Soc.* **2023**, *145*, 6045.
[28] L. Maier, *Helv. Chim. Acta* **1969**, *52*, 827.
[29] D. B. Trowbridge, G. L. Kenyon, *J. Am. Chem. Soc.* **1970**, *92*, 2181.
[30] S. D. Taylor, F. Mirzaei, S. L. Bearne, *Org. Lett.* **2006**, *8*, 4243.
[31] M. G. Mohsen, D. Ji, E. T. Kool, *Nucleic Acids Res.* **2019**, *47*, 9495.
[32] C. Azevedo, J. Singh, N. Steck, A. Hofer, F. A. Ruiz, T. Singh, H. J. Jessen, A. Saiardi, *ACS Chem. Biol.* **2018**, *13*, 1958.
[33] J. Singh, N. Steck, D. De, A. Hofer, A. Ripp, I. Captain, M. Keller, P. A. Wender, R. Bhandari, H. J. Jessen, *Angew. Chem. Int. Ed.* **2019**, *58*, 3928.
[34] a) Y. Kowada, H. Adachi, T. Minami, *J. Phys. Chem.* **1993**, *97*, 8989; b) E. Thilo in *Advances in Inorganic Chemistry and Radiochemistry* (Eds.: H. J. Emeléus, A. G. Sharpe), Academic Press, **1962**, pp. 1–75; c) T. Schneider, K. Schwedtmann, J. Fidelius, J. J. Weigand, *Nat. Synth.* **2023**, 1.
[35] H. J. Jessen, T. Dürr-Mayer, T. M. Haas, A. Ripp, C. C. Cummins, *Acc. Chem. Res.* **2021**, *54*, 4036.
[36] J. R. Van Wazer, K. A. Holst, *J. Am. Chem. Soc.* **1950**, *72*, 639.
[37] a) H. C. Schröder, W. E. G. Müller, *Inorganic polyphosphates. Biochemistry, Biology, Biotechnology*, Springer, Berlin, Heidelberg, **1999**; b) T. Glonek, R. A. Kleps, J. R. Van Wazer, T. C. Myers, *Bioinorg. Chem.* **1976**, *5*, 283.
[38] a) T. Hunter, *Philos. Trans. R. Soc. London Ser. B* **2012**, *367*, 2513; b) J.-M. Kee, T. W. Muir, *ACS Chem. Biol.* **2012**, *7*, 44.
[39] T. Dürr-Mayer, D. Qiu, V. B. Eisenbeis, N. Steck, M. Häner, A. Hofer, A. Mayer, J. S. Siegel, K. K. Baldridge, H. J. Jessen, *Nat. Commun.* **2021**, *12*, 5368.
[40] a) H. Yoshida, Y. Asatsu, Y. Mimura, Y. Ito, J. Ohshita, K. Takaki, *Angew. Chem. Int. Ed.* **2011**, *50*, 9676; b) E. Wolthuis, B. Bouma, J. Modderman, L. Sytsma, *Tetrahedron Lett.* **1970**, *11*, 407.
[41] a) C. E. McKenna, M. T. Higa, N. H. Cheung, M.-C. McKenna, *Tetrahedron Lett.* **1977**, *18*, 155; b) C. M. Sevrain, M. Berchel, H. Couthon, P. A. Jaffrès, *Beilstein J. Org. Chem.* **2017**, *13*, 2186.
[42] S. Lazar, G. Guillaumet, *Synth. Commun.* **1992**, *22*, 923.
[43] J. Vepsäläien, H. Nupponen, E. Pohjala, *Tetrahedron Lett.* **1993**, *34*, 4551.
[44] a) L. Maier, *Angew. Chem. Int. Ed.* **1968**, *7*, 384; b) H.-O. Henning, G. Hilgetag, *Z. Chem.* **1967**, *7*, 169.
[45] G. de Nanteuil, A. Benoist, G. Rémond, J.-J. Descombes, V. Barou, T. J. Verbeuren, *Tetrahedron Lett.* **1995**, *36*, 1435.
[46] M. Simon, K. Karaghiosoff, P. Knochel, *Org. Lett.* **2018**, *20*, 3518.
[47] L. Maier, *Helv. Chim. Acta* **1969**, *52*, 845.
[48] L. Maier, *Angew. Chem. Int. Ed.* **1968**, *7*, 385.
[49] M. Mangstl, V. R. Celinski, S. Johansson, J. Weber, F. An, J. Schmedt Auf der Günne, *Dalton Trans.* **2014**, *43*, 10033.
[50] M. Larsen, R. Willett, R. G. Yount, *Science* **1969**, *166*, 1510.
[51] T. M. Haas, B.-J. Laventie, S. Lagies, C. Harter, I. Prucker, D. Ritz, R. Saleem-Batcha, D. Qiu, W. Hüttel, J. Andexer, B. Kammerer, U. Jenal, H. J. Jessen, *Angew. Chem. Int. Ed.* **2022**, e202201731.
[52] T. Glonek in *Phosphorus Chemistry Directed Towards Biology* (Ed.: W. J. Stec), Pergamon Press, New York, **1980**, pp. 157–174.
[53] J. Singh, A. Ripp, T. M. Haas, D. Qiu, M. Keller, P. A. Wender, J. S. Siegel, K. K. Baldridge, H. J. Jessen, *J. Am. Chem. Soc.* **2019**, *141*, 15013.
[54] a) T. Glonek, J. R. Van Wazer, R. A. Kleps, T. C. Myers, *Inorg. Chem.* **1974**, *13*, 2337; b) T. Glonek, J. R. Van Wazer, T. C. Myers, *Bioinorg. Chem.* **1971**, *1*, 23.
[55] L. Maier, *Helv. Chim. Acta* **1969**, *52*, 858.
[56] N. Qi, N. Zhang, S. R. Allu, J. Gao, J. Guo, Y. He, *Org. Lett.* **2016**, *18*, 6204.
[57] L. Maier, *Helv. Chim. Acta* **1970**, *53*, 1948.
[58] A. F. Kerst, *Patent US 3,646,133* **1972**.
[59] T. Glonek, T. C. Myers, J. R. Van Wazer, *J. Am. Chem. Soc.* **1975**, *97*, 206.
[60] L. Maier, *Patent US 3,849,523* **1974**.
[61] E. J. Griffith, R. L. Buxton, *J. Am. Chem. Soc.* **1967**, *89*, 2884.
[62] J. Nesper, A. Reinders, T. Glatter, A. Schmidt, U. Jenal, *J. Proteomics* **2012**, *75*, 4874.
[63] a) H. Köster, D. P. Little, P. Luan, R. Muller, S. M. Siddiqi, S. Marappan, P. Yip, *Assay Drug Dev. Technol.* **2007**, *5*, 381; b) B. Wang, P. Dai, D. Ding, A. Del Rosario, R. A. Grant, B. L. Pentelute, M. T. Laub, *Nat. Chem. Biol.* **2019**, *15*, 141.
[64] B.-J. Laventie, T. Glatter, U. Jenal, *Methods Mol. Biol.* **2017**, *1657*.
[65] B.-J. Laventie, J. Nesper, E. Ahrné, T. Glatter, A. Schmidt, U. Jenal, *J. Visualization* **2015**, e51404.
[66] A. Kornberg, N. N. Rao, D. Ault-Riché, *Annu. Rev. Biochem.* **1999**, *68*, 89.
[67] a) Y. Gurvich, D. Leshkowitz, N. Barkai, *PLoS Biol.* **2017**, *15*, e2002039; b) R. Gupta, S. Laxman, *eLife* **2021**, *10*; c) G.-D. Kim, D. Qiu, H. J. Jessen, A. Mayer, *mBio* **2023**, e0010223; d) R. Wild, R. Gerasimaite, J.-Y. Jung, V. Truffault, I. Pavlovic, A. Schmidt, A. Saiardi, H. J. Jessen, Y. Poirier, M. Hothorn, A. Mayer, *Science* **2016**, *352*, 986; e) M. Hothorn, H. Neumann, E. D. Lenherr, M. Wehner, V. Rybin, P. O. Hassa, A. Uttenweiler, M. Reinhardt, A. Schmidt, J. Seiler, A. G. Ladurner, C. Herrmann, K. Scheffzek, A. Mayer, *Science* **2009**, *324*, 513.
[68] M. J. Penninckx, *FEMS Yeast Res.* **2002**, *2*, 295.
[69] E. Herrero, J. Ros, G. Bellí, E. Cabiscol, *Biochim. Biophys. Acta* **2008**, *1780*, 1217.
[70] A. Guranowski, E. Starzyńska, M. Pietrowska-Borek, J. Jemielity, J. Kowalska, E. Darzynkiewicz, M. J. Thompson, G. M. Blackburn, *FEBS J.* **2006**, *273*, 829.
[71] a) J. Ying, S. Fu, X. Li, L. Feng, P. Xu, Y. Liu, X. Gao, Y. Zhao, *Chem. Commun.* **2018**, *54*, 8598; b) D. Gan, J. Ying, Y. Zhao, *Front. Chem.* **2022**, *10*, 941228.
[72] a) L. Maier, *J. Organomet. Chem.* **1979**, *178*, 157; b) N. A. Kulak, G. Pichler, I. Paron, N. Nagaraj, M. Mann, *Nat. Methods* **2014**, *11*, 319; c) J. Cox, M. Mann, *Nat. Biotechnol.* **2008**, *26*, 1367; d) J. Cox, N. Neuhauser, A. Michalski, R. A. Scheltema, J. V. Olsen, M. Mann, *J. Proteome Res.* **2011**, *10*, 1794; e) S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M. Y. Hein, T. Geiger, M. Mann, J. Cox, *Nat. Methods* **2016**, *13*, 731; f) J. Cox, M. Y. Hein, C. A. Luber, I. Paron, N. Nagaraj, M. Mann, *Mol. Cell. Proteomics* **2014**, *13*, 2513.

Manuscript received: July 26, 2023
Accepted manuscript online: August 30, 2023
Version of record online: October 13, 2023